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REMARKS

Applicant respectfully requests reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow.

This amendment adds, changes and/or deletes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, with an appropriate defined status identifier.

Status of the Claims

Claims 1, 5, 16, 17, and 35 are now pending in this application. Claims 1, 16, and 17 are currently being amended. Support for each amendment is believed obvious from the record and is found within in the specification.

Objection to Claims 16 and 17

The Examiner objects to claims 16 and 17 under 37 C.F.R. 1.75(c) as improper for failing to further limit the subject matter of the previous claim. In their present form, claims 16 and 17 avoid this rejection. Accordingly, Applicant respectfully requests withdrawal of this rejection.

Rejection of Claims 1, 16, and 17 under 35 U.S.C. § 112, first paragraph

Claims 1, 16, and 17 remain rejected under 35 U.S.C. § 112, first paragraph, as allegedly indefinite. Specifically, the Examiner asserts that "the limitation 'a signal sequence'" is indefinite. Office Action at page 3. Applicants respectfully traverse this rejection.

The second paragraph of section 112 requires only that the claims reasonably apprise those skilled in the art of the scope of the claimed invention. *See e.g. Miles Lab, Inc. v. Shandon, Inc.*, 27 U.S.P.Q.2d 1123 (Fed. Cir. 1993), *cert. denied*, 510 U.S. 1100 (1994), *see*

generally M.P.E.P. § 2173.02. Furthermore, it is the Examiner who has the initial burden of demonstrating that one of skill in the art would not appreciate the metes and bounds of the claimed subject matter. M.P.E.P. § 706.03.

Claims 1, 16 and 17, when read in light of the knowledge of one of ordinary skill in the art, are definite. "Signal sequence" is an art-recognized term defined as:

> Short sequences that direct newly synthesize secretory or membrane proteins to and through membranes of the endoplasmic reticulum, or from the cytoplasm to the periplasm across the inner membrane of bacteria, or from the matrix of mitochondria into the inner space, or from the stroma of chloroplasts into the thylakoid.

THE ENCYCLOPEDIA OF MOLECULAR BIOLOGY (1994) Kendrew and Lawrence, *eds.*, Blackwell Science Ltd., Osney Means, Oxford, p.1019 (copy enclosed) (parantheticals omitted). Numerous signal sequences are known in the art and the physical features and functional characteristics are well-understood. For example, signal sequences are typically located at the N-terminus and are cleaved by signal peptidases after movement across the membrane. *Id.* It also is commonly known that signal sequences possess common structural features – a hydrophobic core, comprising at least eight uncharged residues flanked by a polar basic region on the N-terminal side and a hydrophobic region of about six residues ending with a small uncharged residue. This uncharged residue is the site of peptidase cleavage. *Id.* Accordingly, one of skill in the art would readily understand the scope of the claimed subject matter.

The Examiner inquires about the exclusion of a signal peptide from claim 1 and the inclusion in claim 16. The current form of the claims makes this inquiry moot.

Applicants respectfully request withdrawal of the rejection of claims 1, 16 and 17.

Rejection of Claim 1 under 35 U.S.C. § 112, first paragraph

Claim 1 is newly rejected under 35 U.S.C. § 112, first paragraph as allegedly lacking enablement. Specifically, the Examiner asserts that "[t]he specification does not teach SEQ ID NO:2 lacking the first 13 amino acids has gankyrin biological activity." Office Action at

page 4. The Examiner cites to Wang *et al.* (J. Biol. Chem. 275:507, 2000), Bowie *et al.* Science 247:1306, 1990, Burgess *et al.* (J. Cell Biol. 111:2129, 1990) and Lazar *et al.* (Mol. and Cell Biol. 8:1247, 1988) as purportedly demonstrating that "even a single amino acid substitution or "conservative" amino acid substitution in a protein will often dramatically affect the biological activity and characteristics of a protein." From this the Examiner concludes, "Considering the state of the art and the limited teachings of the specification, it is concluded that undue experimentation would be required to practice the invention." Applicant respectfully request withdrawal of this rejection because it is improper.

The evidence and explanation of record does not establish that claim 1 is not enabled. The Manual of Patent Examining Procedure, TRAINING MATERIALS FOR EXAMINING PATENT APPLICATIONS WITH RESPECT TO 35 U.S.C. SECTION 112, FIRST PARAGRAPH – ENABLEMENT CHEMICAL/BIOTEHCNICAL APPLICATIONS states:

> The Office must accept as being true the statements supporting enablement unless there is an objective reason, usually supported with documentary evidence, to question them, *i.e.*, the burden is on the Office to demonstrate that there is an objective reason, usually supported by documentary evidence, to question the statement.

Example D. Accordingly, the Examiner must provide an objective reason that the claimed polypeptide, namely a polypeptide consisting of an amino acid sequence from Ala at position 14 to Gly at position 226 of SEQ ID NO: 2, does not have a biological activity of gankyrin. Furthermore, such an assertion should be supported by documentary evidence.

The invention of claim 1 provides a "polypeptide consisting of an amino acid sequence from Ala at position 14 to Gly at position 226 of SEQ ID NO: 2 and having biological activity of gankyrin." Indeed, the Applicant has discovered that an amino acid sequence from Ala at position 14 to Gly at position 226 of SEQ ID NO: 2 has a biological activity of gankyrin. As acknowledged by the Examiner, the specification discloses several biological activities of gankyrin, enhancement of colony formation, tumorigenic properties and suppression of apoptosis induction. *See* Example 4. The specification also discloses methods of assaying biological activities of gankyrin. *Id.*

The Examiner provides no objective reason and certainly no documentary evidence to support her assertion that claim 1 is not enabled. Accordingly, Applicant respectfully requests withdrawal of this rejection.

Rejection of Claims 5 and 35 under 35 U.S.C. § 102

Claims 5 and 35 remain rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Kato *et al.* The Examiner asserts:

> Since the protein of art is 100 % identical to instant SEQ ID NO:2 and the specification at Example 4 (pages 55-58) discloses that the various biological activities of gankyrin listed in instant claim 5 is the characteristics of the SEQ ID NO:2, which is encoded by the 678-bp cDNA isolated in Example 1 (pages 45-48), the claims are anticipated by Kato *et al.*

Office Action at page 6.

The evidence and explanation of record does not establish that Kato *et al.* anticipates the instant claims. Kato *et al.* is cited as disclosing the polypeptide of SEQ ID NO: 2. Claim 5 recites a "purified polypeptide that is encoded by a DNA capable of hybridizing under stringent conditions to a DNA having the nucleotide sequence as set forth in SEQ ID NO:1 and that has a biological property of gankyrin selected from the group consisting of an enhancement in the ability of colony formation, a tumorigenic property and a suppression of apoptosis induction, wherein said stringent conditions are defined as washing said hybridized DNA at 50 oC, with 2xSSC and 0.1% SDS". Claim 35 recites a "purified polypeptide that is encoded by a DNA capable of hybridizing under stringent conditions to a DNA having the nucleotide sequence as set forth in SEQ ID NO: 1 and that has the biological properties of gankyrin, wherein said stringent conditions are defined as washing said hybridized DNA at 65oC, with 0.1xSSC and 0.1% SDS."

According to the court in *In re Deuel*, 51 F.3d 1552 (Fed. Cir. 1995), "knowledge of a protein does not give one a conception of a particular DNA encoding it." It is well-known that each amino acid is encoded by a three nucleotide codon. *See* THE ENCYCLOPEDIA OF MOLECULAR BIOLOGY, *supra*, p.215 (copy enclosed). This degeneracy of the genetic code

results in each amino acid being encoded by more than one different codon. *Id.* Nucleic acid hybridization exploits the ability of complementary nucleic acid sequences to hydrogen bond to each other. *Id.* at pp. 503-504 (copy enclosed. The ability of one nucleotide to hybridize to another is a function of the nucleic acid sequence of a given nucleotide. *Id.* The degeneracy of the genetic code can result in a number of nucleotides, each of which can encode the same polypeptide, but lack sufficient similarity to allow for hybridization. Therefore, the evidence and explanation of record does not establish that Kato *et al.* discloses a purified polypeptide that is encoded by a DNA capable of hybridizing under stringent conditions to a DNA having the nucleotide sequence as set forth in SEQ ID NO:1 and that has a biological property of gankyrin selected from the group consisting of an enhancement in the ability of colony formation, a tumorigenic property and a suppression of apoptosis induction, wherein said stringent conditions are defined as washing said hybridized DNA at 50 °C, with 2xSSC and 0.1% SDS as recited in claim 5.

The evidence and explanation of record also does not establish that Kato *et al.* discloses a purified polypeptide that is encoded by a DNA capable of hybridizing under stringent conditions to a DNA having the nucleotide sequence as set forth in SEQ ID NO: 1 and that has the biological properties of gankyrin, wherein said stringent conditions are defined as washing said hybridized DNA at 65°C, with 0.1xSSC and 0.1% SDS as recited in claim 35.

Accordingly, Applicant respectfully requests withdrawal of this rejection.

Rejection of Claims 16 and 17 under 35 U.S.C. § 103

Claims 16 and 17 remain rejected under 35 U.S.C. § 103(a) as allegedly obvious over Kato *et al.* in view of Zhang *et al.* and Jamasa *et al.* Specifically, the Examiner asserts that claims 16 and 17 read on the protein taught by Kato *et al.* In their current form, amended claims 16 and 17 avoid this rejection.

Accordingly, Applicant respectfully requests reconsideration and withdrawal of the rejection.

Conclusion

Applicant believes that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date May 4, 2004

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Enclosure: THE ENCYCLOPEDIA OF MOLECULAR BIOLOGY (1994) Kendrew and Lawrence, *eds.*, Blackwell Science Ltd., Osney Means, Oxford, pp.215, 503-506, and 1019 (copy enclosed)

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segments the heptameric signal sequences are fused to each other giving rise to a signal joint which does not form part of the final coding sequence of the assembled gene. The crossover site in the signal joint is precisely at the heptamer borders.

signal peptidase Integral membrane enzyme of the ENDOPLASMIC RETICULUM membrane which cleaves N-terminal SIGNAL SEQUENCES on proteins as they emerge into the lumen of the endoplasmic reticulum. The enzyme solubilized with neutral detergents requires phospholipid to be active. Its mechanism is unknown, and as a proteinase it is in a class of its own as none of the typical group-specific reagents inhibit it. The purified complex from hen oviduct consists of a protein of M_r 19 000 and a differentially glycosylated form of the same protein (M_r 22 000/24 000), and from canine pancreas, four protein subunits of M_r 25 000, 21 000, 18 000, and 12 000 as well as a glycosylated form (M_r 22 000/23 000). The 21 000 and 18 000 subunits are homologous to the Sec11 protein in yeast; in the *sec11* strain uncleaved secreted proteins accumulate at the nonpermissive temperature. *See also*: PROTEIN TARGETING; PROTEIN TRANSLOCATION.

Shelness, G.S. & Blobel, G. (1990) *J. Biol. Chem.* 265, 9512–9519.

signal sequences, signal peptides Short sequences that direct newly synthesized secretory or membrane proteins to and through membranes of the ENDOPLASMIC RETICULUM, or from the cytoplasm to the periplasm across the inner membrane of bacteria (*see* BACTERIAL ENVELOPE: BACTERIAL PROTEIN EXPORT), or from the matrix of MITOCHONDRIA into the inner space, or from the stroma of CHLOROPLASTS into the thylakoid. They are often, but not universally, in an N-terminal location and are cleaved off by SIGNAL PEPTIDASES after the protein has crossed the membrane. Signal sequences are not homologous, but contain three common structural features — a hydrophobic core, known as the h-region, comprising at least eight uncharged residues flanked by a polar basic region (n-region) on the N-terminal side, and a hydrophilic region (c-region) of about six residues terminating at a small uncharged residue. This residue contributes the carboxyl group of the peptide bond that is cleaved by signal peptidase. The signal sequence is also known as the leader peptide or translated leader sequence. Fusing such a sequence (e.g. from the YEAST MATING FACTOR MFα) to a gene that is to be expressed in a heterologous host ensures secretion of the recombinant protein from the cells (*see* GENETIC ENGINEERING). *See also*: PROTEIN SECRETION; PROTEIN TARGETING; PROTEIN TRANSLOCATION.

von Heijne, G. (1985) *J. Mol. Biol.* 184, 99–105.

signal recognition particle (SRP) A rod-shaped RIBONUCLEOPROTEIN containing a 7SL RNA of 300 nucleotides and six protein subunits of M_r 72 000, 68 000, 54 000, 19 000, 14 000, and 9 000. It targets proteins containing SIGNAL SEQUENCES to the ENDOPLASMIC RETICULUM membrane. SRP binds signal sequences in nascent chains as they emerge from the ribosome and halts or slows elongation until contact is made with DOCKING PROTEIN in the membrane. The 19K subunit mediates binding of the 54K subunit to the 7SL RNA backbone, the 14/9K heterodimer mediates elongation arrest and the 68/72K dimer binds to docking protein. The 54K subunit possesses a consensus GTP-binding domain in its N-terminal section, and a methionine-rich C-terminal domain which folds into several amphiphilic sequences and binds the signal sequence. GTP binding and/or hydrolysis may be required to allow the signal sequence to leave SRP and insert into the membrane before translocation. SRP is isolated by affinity chromatography of salt extracts of mammalian MICROSOMES, and homologous components are found in yeast and bacteria. *See also*: PROTEIN TARGETING; PROTEIN TRANSLOCATION.

Bernstein, H.D et al. (1989) *Nature* 340, 482–486.

signal transduction The process by which the information contained in an extracellular physical or chemical signal (e.g. a hormone or GROWTH FACTOR) is received at the cell by the activation of specific RECEPTORS and conveyed across the plasma membrane, and along an intracellular chain of signalling molecules, to stimulate the appropriate cellular response. *See*: CELL-SURFACE RECEPTORS; OLFACTORY TRANSDUCTION; SECOND MESSENGER PATHWAYS and cross-references therein; VISUAL TRANSDUCTION.

signalling receptors Those RECEPTORS whose activation by their cognate ligand or physical stimulus (e.g. light) leads to the transduction of a signal and the activation of the intracellular biochemical response pathways. *See*: CELL-SURFACE RECEPTORS; SECOND MESSENGER PATHWAYS and cross-references therein.

silencer Name given to presumed protein factors that negatively control expression of eukaryotic genes, and also to the DNA sequences involved in such repression.

silent allele NULL ALLELE.

silent mutation A change in a DNA sequence which does not result in a detectable phenotypic change. Mutations in STRUCTURAL GENES are silent if they do not change the amino acid inserted or if they result in substitution of a residue that does not affect protein function. Silent mutations can also be located outside protein-coding regions. *See*: MOLECULAR EVOLUTION; MOLECULAR PHYLOGENY; MUTATION.

silica gel A desiccant used for the storage of hygroscopic chemicals.

simian virus 40 (SV40) *See*: PAPOVAVIRUSES.

simple-sequence DNA DNA composed of tandemly repeated short noncoding sequences, which occurs within the genomes of most eukaryotes, often in thousands of copies. *See*: GENOME ORGANIZATION; HIGHLY REPETITIVE DNA; SATELLITE DNA; VARIABLE NUMBER TANDEM REPEATS.

simulated annealing A method used in the REFINEMENT OF MACROMOLECULAR STRUCTURES, which has a much larger radius of

ion process results in a coding joint and a SIGNAL JOINT which :s not form part of the rearranged coding sequence. The ıction between assembled V, (D) and J segments is imprecise. ses may be added or removed at the joint (*see* N-REGION /ERSITY).

ding sequence Any DNA or RNA sequence that encodes netic information, that is, the amino acid sequence of a protein the nucleotide sequence of an RNA.

›ding strand The strand of an RNA or DNA molecule that ıntains the genetic (sense) information. Within a single double-randed molecule such as a chromosome the coding strand may ffer for different genes. *See*: GENETIC CODE; TRANSCRIPTION; RANSLATION.

odominance Situation where neither of two different alleles at a ›cus is dominant over the other, usually producing a phenotype ifferent from, and sometimes intermediate between, the two omozygous parental types. *See also*: MENDELIAN INHERITANCE; IUTATION.

:odon A sequence of three successive bases in nucleic acid (DNA ınd RNA) that specifies a particular amino acid or a translation ermination signal (*see* GENETIC CODE). A codon formally refers to he sequence in RNA; the corresponding sequence in DNA is ›ften known as a 'triplet'. The genetic code contains 64 codons of which 61 define one or other of the 20 amino acids known in proteins. The remaining three codons encode signals for the termination of translation. *See also*: PROTEIN SYNTHESIS.

codon bias, codon usage The propensity to use a particular codon to specify a particular amino acid. The GENETIC CODE is redundant, with 61 codons carrying the information for 20 different amino acids. Most amino acids are encoded by more than one codon; leucine, for example, has six different codons (the maximum number) — UUA, UUG, CUU, CUC, CUG, CUA. Analysis of DNA sequences from a wide variety of organisms has revealed that not all codons for a specific amino acid are used with equal frequency. Such bias in codon usage is both species specific and, within a given species, is more extreme for mRNAs that encode abundant proteins. Furthermore, the codon bias observed for a given species directly relates to the abundance of the corresponding isoacceptor tRNA species; the more frequently a codon is used, the more abundant the tRNA used to decode that codon. Codon bias may represent a means of ensuring optimal translational efficiency of mRNAs coding abundant proteins.

Ikemura, T. (1982) *J. Mol. Biol.* 158, 573–597.

coelenterate development The coelenterates comprise the hydroids, which are usually colonial (although the well-known *Hydra* itself is a free-living polyp), together with the jellyfish and sea anenomes. The latter have no developmental biology, save for some striking examples of metaplasia during regeneration. Following the celebrated work of Tremblay in the 18th century, the hydroids have been intensively studied from the point of view of pattern regeneration and the results have had a far-reaching influence on developmental biology. Work on colonial hydroids by Child contributed to the concept of developmental GRADIENTS, the later work of Rose to the law of distal transformation and the still later work of Wolpert to the theory of POSITIONAL INFORMATION.

In *Hydra* there seem to be separate processes controlling regeneration of the head and the foot, but the rules for the two extremities are quite similar. If the head is grafted into the flank it can suppress regeneration from the cut surface. The characteristics of this interaction suggest that the head produces a diffusible inhibitor. If the normal head is removed then the inhibition is quickly lost and the tissue near the cut autonomously acquires the positional coding of a new head. A short segment from the midbody region may regenerate a head (or a foot) at both ends, to produce a 'bipolar form'. Various substances have been isolated from *Hydra* and other coelenterates that accelerate or retard regeneration from one or other extremity, and it is also known that substances interfering with signal transduction mechanisms can have severe effects on polarity and pattern.

The advantage of *Hydra* is that the microsurgery is quite easy, and that long-range inductive signalling appears to persist throughout the life of the animal. Colonial hydroids in particular seem to secrete MORPHOGENS in the medium so are favourable material for morphogen identification.

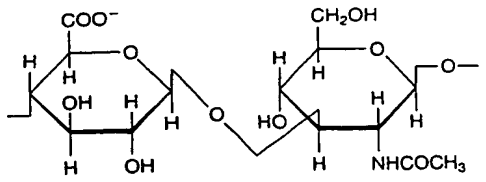
coelom The cavity between SOMATOPLEURE and SPLANCHNOPLEURE, the two layers of cells derived from the LATERAL PLATE MESODERM. It characterizes higher animals.

cofilin *See*: ACTIN-BINDING PROTEINS.

cohesive ends Sometimes referred to as 'sticky ends', these are the single-stranded extensions found in double-stranded DNA which has been digested with a RESTRICTION ENZYME which cuts in a staggered fashion. The single-stranded 5′ or 3′ extensions produced by a given restriction enzyme are complementary and thus can base pair via nominal hydrogen bonding (Fig. C58). Single-stranded extensions generated by different restriction enzymes are not usually complementary although there are some exceptions to this rule.

coiled coil Protein conformation found, for example, in collagen and laminin (*see* EXTRACELLULAR MATRIX MOLECULES) in which a stretch of α-helix is further helically coiled.

cointegrating plasmid A vector used in the cointegration method of gene transfer in plants. The vector is based on a standard *Escherichia coli* CLONING VECTOR (e.g. pBR322) which also carries a T-DNA region from the TI PLASMID of *Agrobacterium*. The 'foreign' gene to be introduced into the plant is inserted into this plasmid, which is then transferred via CONJUGATION or TRANSFORMATION into an *Agrobacterium* strain carrying an intact Ti plasmid. Recombination between the recombinant plasmid and the endogenous Ti plasmid occurs by way of the homologous T-DNA sequences, generating a new form of the Ti plasmid now carrying the required gene. Subsequent infection of the plant with



$(\text{-3GlcNAc(p)}\beta\text{1,4GlcA(p)}\beta\text{1-})_n$. The only variation is in chain length, which is usually $>10^4$ residues. Hyaluronic acid is a product of some bacterial and animal cells. In the latter, it is a major component of many connective tissue matrices, where it interacts with the core protein and 'link protein' of PROTEOGLYCAN to form large proteoglycan aggregates. In mammals, hyaluronic acid is synthesized at the cell surface by successive transfers from UDP-GlcNAc + UDP-GlcA, without specific priming, as a 'giant SUGAR NUCLEOTIDE' from which it is probably released by 5′-nucleotidase. Two transferases act alternately. Synthesis is by addition at the reducing terminal, unlike other animal glycans (but like many bacterial glycans). Hence, it should not be classified as a GLYCOSAMINOGLYCAN, as formerly. *See also*: EXTRACELLULAR MATRIX MACROMOLECULES.

Prehm, P. (1983) *Biochem. J.* 211, 191–198.

hyaluronidase Hyaluronidase (EC 3.2.1.35) splits (β1,4)-*N*-acetylglucosaminide links in HYALURONIC ACID and other glycoconjugates. The enzyme is found in the LYSOSOMES of many mammalian tissues but also extracellularly in body fluids. The lysosomal hyaluronidase has a sharp pH optimum in the range pH 3.5–4.1, distinguishing it from testicular hyaluronidase (deriving from the acrosomes of spermatozoa) which has a broad pH optimum extending up to pH 6.0.

hybrid antibodies Artificially constructed ANTIBODY molecules having two, or more, antigen-binding sites of differing specificities. Hybrid antibodies may be produced following reduction and reoxidation of a mixture of antibodies of different specificities, or of their $F(ab')_2$ fragments. Only a proportion of the oxidized products will be hybrid antibodies but they can be purified by appropriate immunoaffinity techniques. Hybrid antibodies may also be produced following the fusion of two HYBRIDOMA cell lines that produce antibodies of differing specificity. Random association of light and heavy chains results in the formation of several antibody populations. Hybrid antibodies of the required dual specificity can be isolated by immunoaffinity techniques.

hybrid-arrested translation A technique used to identify and isolate the protein product of a cloned gene. The cloned double-stranded DNA is denatured and annealed to a mixture of mRNAs known to contain mRNA encoded by the required gene. mRNA : DNA hybrids are formed, which renders the relevant mRNA unavailable for translation. Translation of the annealed mixture in an IN VITRO TRANSLATION system thus results in the absence of the translation product of the test gene. Comparison with the translation products of an untreated sample can then identify the missing protein.

hybrid dysgenesis *See*: P ELEMENT.

hybrid hybridomas Hybridomas produced by the fusion of two hybridomas that express unique properties dependent on the combined characteristics of the parent hybridomas. They may be produced following T–T cell or B–B cell fusions.

hybrid nucleic acid A double-stranded NUCLEIC ACID in which the two strands are from different sources.

hybrid promoter A functional transcriptional PROMOTER containing DNA sequence elements from two (or more) well characterized promoters. Such hybrid promoters are constructed *in vitro* and are used to either probe the role of specific *cis*-acting DNA sequences in the control of TRANSCRIPTION, or to optimize the efficiency and/or regulation of the original promoter. An example of the former would be the various mammalian hybrid promoters which contain ENHANCER-like elements from the SV40 promoter. A well studied example of the latter is the *tac* promoter from *Escherichia coli* which contains the −35 region of the *trp* promoter and the −10 region of the *lac* promoter, and is widely used to express HETEROLOGOUS genes in *E. coli*.

hybrid-release translation A technique used to identify and isolate the protein product of a cloned gene. It is the converse of HYBRID-ARRESTED TRANSLATION but has the same overall objective. The cloned DNA is denatured and attached to a matrix of, for example, NITROCELLULOSE. A complex mixture of mRNAs is then added and hybrid mRNA : DNA allowed to form. Unannealed mRNAs are then removed by washing and the annealed mRNA released from the hybrid by denaturation. This mRNA is then translated in an IN VITRO TRANSLATION system to identify its protein product.

Hybridization

NUCLEIC ACID hybridization is a powerful and widely used technique which exploits the ability of complementary sequences in single-stranded DNAs or RNAs to pair with each other to form a double helix. Hybridization can take place between two complementary DNA sequences, between a single-stranded DNA and a complementary RNA, or between two RNA sequences. The two polynucleotide strands are held together in an antiparallel configuration by hydrogen bonding between the bases G and C and between A and T (or U) (*see* BASE PAIR). The structure is stabilized by base-stacking interactions (*see* DNA; NUCLEIC ACID STRUCTURE; RNA). The reformation of a double-stranded DNA (or RNA) from its two original single strands is also known as reannealing or renaturation.

Hybridization can occur between two separate strands (intermolecular hybridization), or between inverted repeat sequences within a single strand of nucleic acid (intramolecular hybridization; *see* HAIRPIN; PALINDROME). The helix-to-coil transition that occurs as the double helix is denatured can be monitored by

measuring the absorbance at 260 nm, as the absorption of light at this wavelength by single-stranded nucleic acid is greater than by double-stranded nucleic acid (*see* HYPERCHROMIC EFFECT).

Nucleic acid hybridization has been exploited in a wide range of experimental procedures to address many questions about the synthesis, structure and function of DNA and RNA.

Stability

The thermodynamic stability of hybridized sequences (hybrids) is expressed in terms of the temperature at which the strands separate (the melting, or transition temperature, T_m). The T_m (expressed in °C) is dependent on several factors which are divisible on the one hand into those relating to the nucleotide sequences involved in the hybridization, and on the other into the experimental conditions under which the hybridization takes place. The effect of the (G + C) content of DNA on the T_m of a hybrid in a solution containing 0.2 M Na^+ is given by the following equation [1]:

$$T_m = 69.3 + 0.41 \text{ (percentage (G + C) content)}$$

The effect of ionic strength (I) upon the T_m is given by the following equation [2]:

$$T_{m2} - T_{m1} = 18.5 \log_{10} (I_2/I_1)$$

Hybridization of similar but not identical sequences

Nucleic acid hybridization may be used to measure the degree of similarity (degree of HOMOLOGY) between two nucleic acid sequences as hybridization can occur between sequences which contain some complementarity, but which are not identical; that is, hybrids can tolerate mismatched base pairs (mismatches) in the double helix. DNA : DNA hybridization of this sort has been used to measure the broad similarity between different genomes for taxonomic and evolutionary studies (*see* MOLECULAR PHYLOGENY).

The degree of sequence homology between the two strands in the hybrid influences the stability of the hybrid. The T_m of duplex (double-stranded) DNA decreases by 1°C for every 1–1.5% of mismatches [3] but this relationship is dependent on the distribution of mismatches thoughout the sequences. The above equations assume that the length of the DNA is not less than about 150 base pairs, and therefore, an empirical guide has been developed to estimate the stability of hybrids formed by short DNA sequences (for example synthetic OLIGONUCLEOTIDES) of between 11 and 23 bases long in 1 M Na^+ [4]:

$$T_m \text{ (in °C)} = 2(\text{number of A + T residues}) + 4(\text{number of G + C residues})$$

The stability of hybrids also depends on whether DNA or RNA is involved in the hybridization:

$$T_m \text{ of RNA : RNA} > \text{RNA : DNA} > \text{DNA : DNA}$$

pH has relatively little effect on T_m in the range pH 5–9, but the molarity (M) of Na^+ influences hybrid stability significantly, and hybridization conditions can be manipulated by the addition of formamide which destabilizes hybrids [5]:

$$T_m = 81.5 + 16.6 \log_{10} [M] + 0.41 \text{ (\% content of G + C)} - 500/\text{length} - 0.62 \text{ (\% of formamide)}$$

Hybridization conditions which allow only the hybridization of identical or very similar sequences are known as stringent conditions; these include high temperature, low ionic strength and high formamide concentrations. Those that allow the hybridization of less similar sequences are known as relaxed conditions; they include lower temperatures, ionic strengths and low or zero formamide concentrations.

Solution hybridization

The rate at which hybridization occurs depends on the concentration of the nucleic acid sequences capable of forming hybrids. (An exception to this is intramolecular hybridization by INVERTED REPEAT sequences, the renaturation of which is independent of concentration.) This feature has been exploited in the analysis of DNA and RNA sequence complexity (the number of different sequences present and the relative concentration of these sequences) by solution hybridization where all molecules are freely diffusible.

By separating genomic DNA into single strands and then following the kinetics of its renaturation, several broad classes of sequence have been found in the typical eukaryotic genome: (1) highly repetitive DNA (satellite DNA); (2) moderately repetitive DNA; and (3) unique DNA sequences (*see* GENOME ORGANIZATION). The parameter used to measure the kinetics of renaturation is the C_0t value:

C_0t = initial concentration (C_0) in moles multiplied by the time (t) in seconds it takes for the DNA to reanneal (*see* COT ANALYSIS) [6].

Similarly, the analysis of renaturation kinetics for specific RNA populations with synthetic complementary DNA (cDNA) made from the RNA (R_0t values) has allowed the sequence complexity of messenger RNA populations to be determined (i.e. the proportion of the population that is made up of mRNAs of high or low abundance, and the number of sequences present in each abundance class). Solution hybridization, followed by digestion with S1 nuclease or ribonuclease (RNase) A, which under the appropriate conditions cleave only single-stranded DNA or RNA respectively, has been applied to the mapping of TRANSCRIPTION UNITS within cloned DNA sequences (*see* S1 MAPPING). Transcription units can also be mapped at lower resolution by the analysis of RNA : DNA heteroduplexes (double-stranded nucleic acids containing strands of different origin) by electron microscopy (*see* R-LOOP MAPPING).

Filter hybridization

In filter hybridizations, one of the nucleic acid components of the hybridization is immobilized on a membrane filter (originally nitrocellulose, but more recently, charged nylon membranes have also been used).

Southern hybridization (Southern blotting)

This technique, named after its inventor, Ed Southern, enables very low concentrations of specific sequences to be detected within complex DNA populations and is of wide application. Hybridization of a labelled probe directly to DNA fragments embedded in a gel is insensitive. So, after separation of RESTRICTION FRAGMENTS of DNA by ELECTROPHORESIS on agarose gels, they are transferred and fixed to a membrane filter (blotting), and then hybridized with a radioactively labelled DNA or RNA PROBE containing the required sequence [7] (Fig. H19). Among its many applications are the study of RFLPs (restriction fragment linked polymorphisms) in human disease and its use in DNA TYPING.

Northern blotting

This technique is similar to Southern blotting, and was named by analogy, and is used to analyse RNA sequences. RNA is electrophoresed under denaturing conditions on agarose gels, transferred and fixed to the membrane filter, and then hybridized with a radiolabelled DNA or RNA probe [8]. This procedure has been used to analyse specific messenger RNAs within complex RNA populations, and hence to study the differential expression of specific genes in response to particular physiological or environmental stimuli.

Dot blotting

Accurate and rapid measurement of the abundance of specific RNA or DNA sequences is often performed by dot blotting. Here, samples are dotted directly onto the filters without previous separation by electrophoresis. Filters are then treated as for Southern or northern hybridizations.

In situ hybridization

This is used to detect and locate specific DNA or RNA sequences in tissues or on chromosomes. A radioactively or fluorescently labelled DNA or RNA probe of the required sequence is applied to fixed tissue or chromosome preparations, where it hybridizes with any complementary sequences present. Unhybridized probe is then washed off. In the case of radioactively labelled probes, images of probe distribution are then superimposed upon the microscopic images of the chromosomes or tissue sections to determine the distribution of hybridized radioactive probe, and hence the complementary sequences, within the samples. Fluorescently labelled chromosomes, etc. are viewed directly.

In situ hybridization of chromosomes, especially fluorescent *in situ* hybridization (FISH), is widely used to map particular sequences in the genome (*see* HUMAN GENE MAPPING), or to study CHROMOSOME ABERRATIONS. *In situ* hybridization of tissue sections has been used extensively to study tissue-specific gene expression especially in the study of the developmental regulation of genes involved in pattern formation during *Drosophila* embryogenesis (*see* DROSOPHILA DEVELOPMENT).

Hybridization probes

Radioactively labelled DNA probes are frequently synthesized by

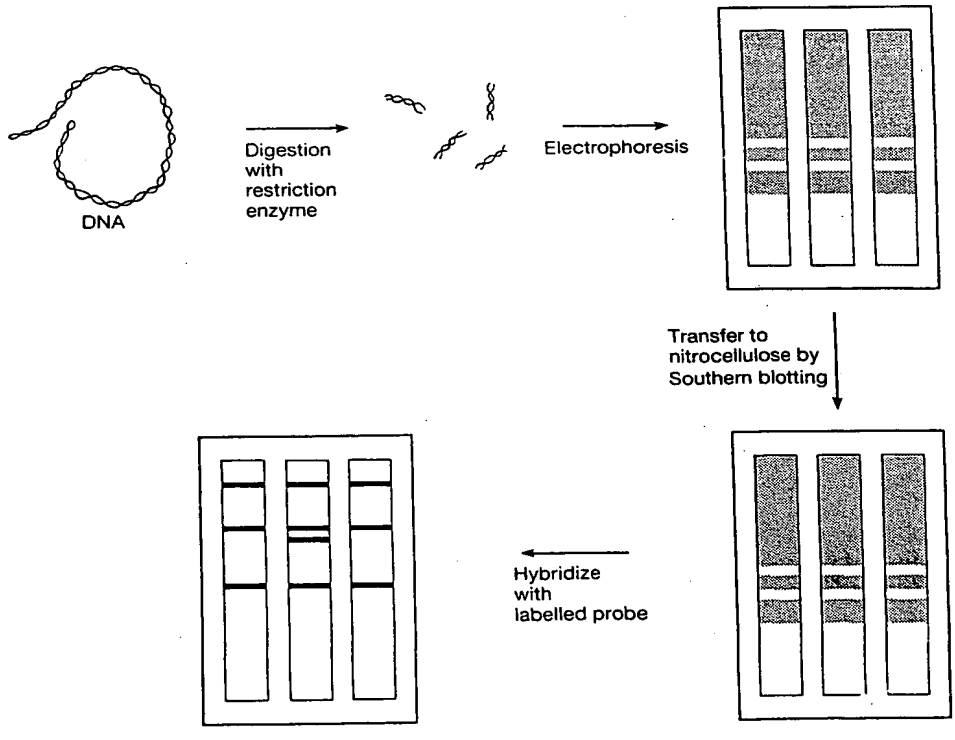


Fig. H19 Southern blotting. DNA fragments are first separated by electrophoresis on a gel which is then placed in contact with a sheet of nitrocellulose (or other suitable membrane) the same size as the gel. An appropriate solution is then drawn through the gel and nitrocellulose, eluting the DNA and trapping it on the nitrocellulose sheet. The DNA on the nitrocellulose sheet is then probed with specific probes. A similar procedure is followed for RNA in northern blotting.

nick-translating, random priming, or end-labelling specific DNA fragments or plasmids (*see* END-LABELLING; NICK TRANSLATION; POLYNUCLEOTIDE KINASE; RANDOM PRIMING). Radioactively labelled RNA probes are often made by *in vitro* transcription of the relevant DNA sequence. The sensitivity of the hybridization techniques described above depends upon the specific activity of the radioactive probe.

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1 Marmur, J. & Doty, P. (1962) Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. Mol. Biol.* 5, 109–118.
2 Dove, W.F. & Davidson, N. (1962) Cation effects on the denaturation of DNA. *J. Mol. Biol.* 5, 444–467.
3 Bonnet, T.I. et al. (1973) Reduction in the rate of DNA reassociation by sequence divergence. *J. Mol. Biol.* 81, 123–135.
4 Schulze, D.H. et al. (1983) Identification of the cloned gene for the murine transplantation antigen H2Kb by hybridization with synthetic origonucleotides. *Mol. Cell. Biol.* 3, 750–755.
5 McConaughy, B.L. et al. (1969) Nucleic acid reassociation in formamide. *Biochemistry* 8, 3289–3295.
6 Adams, R.L.P. et al. (Eds) (1986) *The Biochemistry of the Nucleic Acids*, 10th revised edn (Chapman & Hall, London).
7 Southern, E. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98, 503–517.
8 Thomas, P.S. (1980) Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA* 77, 5201–5205.

hybridoma Clone of cells produced by the fusion of a somatic cell with a cell line that has the capacity to survive and proliferate *in vitro*. Hybridomas derived from the fusion of an antibody-producing B cell from a specifically immunized animal with a PLASMACYTOMA cell line (Fig. H20) were originally developed for the production of MONOCLONAL ANTIBODY of defined specificity. Hybridomas may be stored frozen in liquid nitrogen and recovered when further product is required. T cell hybridomas are generated in a similar manner.

Milstein, K. (1980) *Sci. Am.* 243, 66–74.

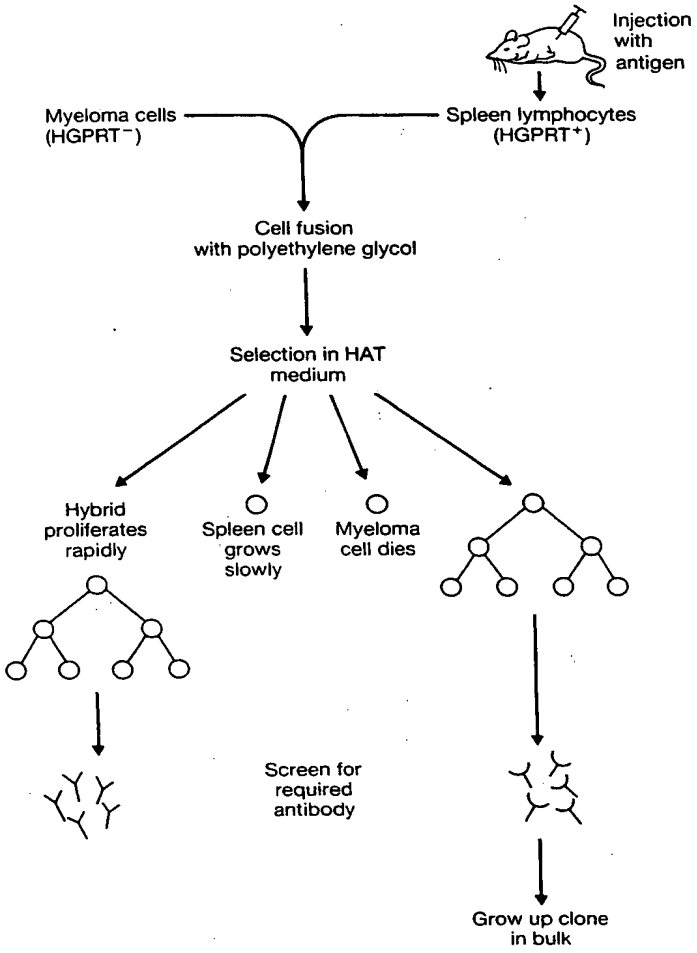

Fig. H20 Production of hybridomas.

***Hydra* development** This small freshwater coelenterate (class Hydrozoa) is used as a simple system in which to study regeneration, and the role of morphogenetic GRADIENTS in development. *Hydra* development involves the twin process of head and foot formation at opposite ends of a tubular body column. As the animal grows, new animals bud off from the side of the adult, in a region called the budding zone. Hydroids have considerable powers of regeneration, and new animals can be generated from fragments as small as 1/50th the size of a normal adult. The head and foot are thought to be specified in development by two pairs of morphogenetic gradients; one gradient of each pair has an activating role, while the other is inhibitory. The 'head gradient' (activating) has its maximal value at the head end of the animal, the 'foot gradient' is maximal at the foot. A piece of donor head tissue will give rise to a new bud when transplanted to the foot of a host animal because it possesses a high amount of 'head activator', whereas transplantation to another head region produces no head induction. The two gradient pairs are used to explain the results of such grafting experiments. To increase the frequency with which a middle piece of body tissue induces a new head, it is first necessary to remove the head region of the host; head induction occurs in this case because the head inhibitor is no longer so abundant. It is thought to be the existence of the two inhibitory gradients that determines the location of the mid-body budding zone. *Hydra* development can be seen as a paradigm of PATTERN FORMATION through morphogenetic gradients.

hydrazinolysis The cleavage of DNA with hydrazine usually as part of the Maxam and Gilbert DNA SEQUENCING method. In the absence of added salt, hydrazine will cleave at both pyrimidine residues (C, T) whereas in the present of salt, cleavage will only occur at C residues.

hydrogen bond Weak noncovalent bond in which a hydrogen atom covalently bonded to a very electronegative atom interacts with another atom. In biological macromolecules the shared hydrogen is covalently linked to an oxygen, a nitrogen or, occasionally, a sulphur atom (the donor), and the other partner (the acceptor) is also oxygen or nitrogen or, occasionally, sulphur. The hydrogen is attracted to the acceptor by a partial negative